Drosophila Dalmatian combines sororin and shugoshin roles in establishment and protection of mitotic cohesion

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Appendix

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Appendix Supplementary Methods

Cell culture, RNAi, and transfection

S2 cells were cultivated at 24 °C in complete media; Schneider's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biowest) and 1% penicillin/streptomycin. The synthesis of dsRNA and RNAi experiments were performed as previously described (Bettencourt-Dias & Goshima, 2009). Briefly, DNA fragments of 500–600 bp were amplified from the S2 cell complementary DNA (cDNA) or genomic DNA by polymerase chain reaction (PCR) introducing T7 RNA polymerase binding sites and transcribed *in vitro*. Primers used are shown in Table S1. Cells numbering 10^6 were treated with 15 μg dsRNA in serum-free medium and cultivated in the presence of 10% FBS for 2–7 days. Transfection of expression vectors was performed by TransIT-Insect transfection reagent (Mirus) according to the manufacturer's protocols.

RPE-1 cells were cultivated at 37 °C in Dulbecco's modified Eagle medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (GIBCO) and 1% penicillin/streptomycin. GFP-tagged constructs were transfected by Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocols. The cells were treated with control or Sgo1 small interfering (siRNAs) 48 h after transfection, and cultured for an additional 24 h as described previously (Nishiyama et al, 2010). After 3 h treatment of nocodazole (330 nM), the cells were subjected to immunofluorescence or chromosome spread.

Chromosome spreads

S2 cells in complete media were hypotonically swollen in 40% complete medium/60% tap water for 5 min at room temperature. The cells were fixed with freshly made Carnoy's solution (75% methanol, 25% acetic acid), and washed in the fixative several times. The fixed cells were dropped onto glass slides and dried at room temperature. The slides were stained with 4′,6-diamidino-2-phenylindole (DAPI)/80% glycerol-PBS solution. For chromosome spread followed by immunofluorescence microscopy, hypotonically swollen cells were spun onto glass slides at 1,000 rpm for 5 min using a cytospin centrifuge (Thermo), pre-extracted and fixed with 4% paraformaldehyde. After blocking with 10% goat serum for 1 h at room temperature, the specimens were subjected to immunofluorescence microscopy. For RPE-1 cells, chromosome spreads were performed as previously described (Nishiyama et al, 2010).

Fluorescence *In Situ* Hybridization (FISH)

For FISH to analyze the distance between two sister chromatids in interphase, *D.melanogaster* Bac clone RP98-13J19 corresponding to chromosome 3R region 92E-92F (chr3R: 20478009-20648947) was labeled with Digoxigenin (DIG) by Nick translation kit (Roche) according to manufacturer's protocol, and used as a probe. Cells grown on poly-L-K-coated

coverslips were fixed in 4% PFA/PBS for 15 min at room temperature and washed in 0.5% Triton-X100/RNaseA/PBS. The fixed cells were treated successively with 0.1M HCl, 2xSSC, 1% PFA, 70% and 100% EtOH. Cells were incubated with the probe at 100ºC for 10 min and at 37ºC overnight and subsequently treated with 0.01% Triton-X100/2xSSC, and anti-DIG Rhodamine. DNA was counterstained with DAPI and the coverslips were mounted with ProLong Gold (Invitrogen).
For mitotic FISH with pH3S10 immunostaining, DNA probes corresponding to pericentromeric ChrX 359bp satellite DNA (GGGATCGTTAGCACTGGTAATTAGCTGC) was labeled with DIG by DIG-Oligonucleotide 3'-End labeling kit (Roche) according to manufacturer's protocol. After 3 h treatment of MG132 and following hypotonic treatment, cells were spun onto slide glasses by using Cytospin (Thermo Fisher Scientific), fixed in 4% PFA/PBS, and incubated with the ChX probe at 37ºC overnight. The cells were incubated with anti-DIG Rhodamine anti-pH3S10, and subsequently with secondary antibody. DNA was counterstained with DAPI and ProLong Gold was mounted on the specimens.

Immunofluorescence microscopy
S2 cells were grown on glass bottom chamber (IWAKI) treated with Concanavalin A. The cells were washed with PBS, fixed with 4% paraformaldehyde (PFA)/PBS for 20 min, and blocked with 10% goat serum. For pre-extraction, cells were pre-extracted by 0.1% Triotn-X100-PBS for 3 min prior to fixation. The specimens were incubated with primary antibodies at 4ºC overnight (for anti-MEI-S332, H3K9me3, and DmtN240 antibodies) or at room temperature for 1 hour (for other antibodies) and secondary antibodies (Alexa 488, 568, and 647; Invitrogen) at room temperature for 1 hour. DNA was counter stained with DAPI and the glass was covered by Fluoromount (Diagnostic BioSystems) for microscopy observation. Images were taken on Nikon Ti-E microscope equipped with a confocal unit CSU-W1 (Yokogawa), an EMCCD camera iXon Ultra 888 (Andor), and 100x CFI Apo TIRF (NA 1.49) objective (Nikon). To image chromosome spreads, Nikon Ni microscope equipped with a CoolSnapHQ2 CCD camera (Photometrics) and 100x CFI Plan Apo Lambda (NA 1.45) objective (Nikon) was used. For RPE-1 cells, immunofluorescence microscopy was performed as previously described (Nishiyama et al, 2010).

Time-Lapse and Photobleaching Microscopy (FRAP)
Cells were grown in glass bottom chamber (IWAKI) and time-lapse microscopy was performed with a 40x CFI Plan Fluor (NA 1.43) objective (Nikon) on Nikon Ti-E microscope or a 100x CFI Plan Apo VC (NA 1.40) objective (Nikon) on Nikon TE-2000 microscope equipped with a confocal unit CSU-X1 (Yokogawa) and an EMCCD camera ImagEM (Hamamatsu).

For fluorescence recovery after photobleaching (FRAP), Dmt-GFP or Dmt-ΔN116-GFP-expressing cells were grown in cover-glass chamber (IWAKI) and live-cell images were acquired on an LSM780-DUO-NLO (Zeiss) confocal microscope using a 63x Plan-Apochromat objective. Selected regions corresponding to about a half area of nuclei were bleached by 488 nm laser. After the bleach, images were taken in every 30 sec (Fig 5 and H2B in Fig EV3B) or 0.3 sec for 15 min or 25 sec, respectively. Signal intensities were measured using ImageJ software at bleached and unbleached regions and normalized. Data were analyzed using Prism 6 software and two-phase association curve fitting ($I = Span^{fast}*(1-exp(-k_{Off}{}^{fast})*time) + Span^{slow}*(1-exp(-k_{Off}{}^{slow})*time)$). Because almost all Dmt proteins are apparently chromatin-bound (Fig 2A), we assumed that there are 2 cellular fractions of Dmt, namely dynamic chromatin-bound fraction and stable chromatin-bound faction, which were corresponding to "fast" and "slow" fractions, respectively in the above equation. Residence time (τ) of the dynamic and stable chromatin-bound fractions of Dmt were calculated as $\tau = 1/k_{Off}$.

Yeast two-hybrid assay

Yeast two-hybrid assay was performed according to Matchmaker system (Clontech). Dmt 86-116^{WT} or 86-116^{VEIE} are cloned into pGBT9 as bait, and HP1a were cloned into pGAD424 as prey. These plasmids were transformed into *Saccharomyces cerevisiae* SFY526 strain that carried the GAL1-lacZ reporter. β-Galactosidase filter assay was performed according to manufacturer's protocols.

Appendix Supplementary Reference

Bettencourt-Dias M, Goshima G (2009) RNAi in Drosophila S2 cells as a tool for studying cell cycle progression. *Methods Mol Biol* **545:** 39-62

Nishiyama T, Ladurner R, Schmitz J, Kreidl E, Schleiffer A, Bhaskara V, Bando M, Shirahige K, Hyman AA, Mechtler K, Peters JM (2010) Sororin mediates sister chromatid cohesion by antagonizing Wapl. *Cell* **143:** 737-749

Appendix Figure Legends

Appendix Figure S1. Dmt is localized to heterochromatin in living cells

(A) Images of living S2 cells stably expressing Dmt-GFP and HP1a-mCherry (HP1-mCh) were taken by N-SIM system (Nikon). Dmt-GFP was co-localized with HP1a-mCherry in living cells. DNA was counterstained with Hoechst 33342. Bar: 5 μm.

(B) Images of living S2 cells stably expressing Dmt-GFP and H2B-mCherry were treated with control or Dmt dsRNA. Dmt RNAi did not apparently affect heterochromatin formation in interphase cells. Bar: 20 μm.

(C) Images of living S2 cells stably expressing Scc1-GFP. Scc1-GFP was not accumulated on heterochromatin and rather uniformly localized in living cell nucleus. DNA was counterstained with Hoechst 33342. Bar: 2 μm.

(D) Time-lapse microscopy of cells stably expressing Dmt-GFP and mCherry-tubulin. Selected frames from a time-lapse series are shown. t = 0 (min) is set to nuclear envelope breakdown (NEBD). Bar: 10 μm.

Appendix Figure S2. Dmt is associated with heterochromatin through its N-terminal region

(A) Dmt-GFP cells were treated with control, Pds5, or San+Deco dsRNAs, pre-extracted prior to fixation, and chromatin-bound Dmt-GFP signals were measured. DNA was counterstained with DAPI. The Dmt-GFP intensities were normalized by DAPI intensities (≥ 45 cells per condition, mean ± SEM).

(B) Cells expressing Dmt-GFP were subjected to control or San+Deco RNAi. DNA was counterstained with DAPI. Heterochromatin localization of Dmt-GFP was not significantly altered in San+Deco RNAi. Bar: 5 μm.

(C) Cells expressing Dmt-GFP and HP1a-mCherry were subjected to control or HP1a+HP1b RNAi. The cells were pre-extracted prior to fixation and DNA was counterstained with DAPI. Bar: 5 μm (upper). Dmt-GFP and HP1a-mCherry intensities were normalized by DAPI intensity and the results from control cells were normalized to 1 (≥ 41 cells per condition, mean ± SEM) (lower).

(D) Cells were treated with control or HP1a+HP1b dsRNAs and their cohesion were examined by FISH (n = 3, ≥ 20 cells per condition, mean ± SEM).

(E) Whole cell extracts from control or HP1a+HP1b RNAi cells were examined by immunoblotting with indicated antibodies.

(F) H2B-mCherry cells co-expressing Dmt-full-, N116-, ΔN116-, or C320-GFP were fixed or pre-extracted prior to fixation and the localization of Dmt-GFP were observed. Although both Dmt-ΔN116- and C320-GFP failed to be accumulated to heterochromatin, Dmt-ΔN116 could still associate with chromatin, whereas Dmt-C320 could not. Bar: 10 μm.

(G) Living H2B-mCherry cells co-expressing Dmt-86-116-, C320-, 86-116-C320-, or full-GFP were imaged to examine the Dmt-GFP localization. Note that heterochromatin accumulation of 86-116-C320 or full length of Dmt was more prominent compared with 86-116. Bar: 10 μm.

Appendix Figure S3. Dmt-full^{VEIE}-GFP is localized on heterochromatin in S2 cells

(A) Dmt-full^{WT}- or Dmt-full^{VEIE}-GFP was expressed in S2 cells and the localization of Dmt-GFPs were examined. DNA was counterstained with DAPI. Magnified images of a chromosome are shown on the right. Bar: 5 μm (unmagnified) and 1 μm (magnified).

(B) Whole cell extracts from S2 cells expressing Dmt-GFP and/or Dmt-mCherry were subjected to GFP-nanobody pull-down assay. The whole cell extracts (input) and pull-down fractions were immunoblotted with anti-GFP antibody (Dmt-GFP) and anti-mCherry antibody (Dmt-mCherry).

Appendix Figure S4. Pds5 is required for cohesin association with Dmt

(A) S2 cells or Dmt-GFP-expressing cells were treated with control, Deco, or Pds5 dsRNA. Whole cell extracts from these cells were subjected to GFP-nanobody pull-down assay. The whole cell extracts (input) and pull-down fractions were immunoblotted for indicated proteins. Dmt-GFP was detected by anti-GFP antibody.

(B and C) Whole cell extracts from untransfected S2 cells or cells transfected with Pds5-mCherry and variously truncated Dmt-GFP were subjected to GFP-nanobody pull-down assay. The whole cell extracts (input) and/or pull-down fractions were immunoblotted with anti-mCherry and anti-GFP antibodies. Asterisks indicate nonspecific signals.

(D) Whole cell extracts from untransfected S2 cells (-) or cells transfected with Dmt (WT)- or Dmt (ΔCPB)-GFP were subjected to GFP-nanobody pull-down assay. The whole cell extracts (input) and pull-down fractions were immunoblotted with anti-Scc1 and anti-GFP antibodies.

(E) Untransfected S2 cells (-) or Dmt (WT)- or Dmt (ΔCPB)-GFP-transfected cells were treated with control or Dmt dsRNA. Whole cell extracts from these cells were subjected to GFP-nanobody pull-down assay and the pull-down fractions were immunoblotted with Dmt and tubulin antibodies. Note that both WT and ΔCPB were overexpressed compared with endogenous level.

Appendix Figure S5. PP2A is required for sister-chromatid cohesion

(A) Cells expressing 87B (PP1-C)-mCherry were spun onto slide glasses after hypotonic treatment and immunostained with anti-mDsRed (87B-mCh) and anti-Dmt antibodies. DNA was counterstained with DAPI. Higher-magnification images are shown in the inserts. Bar: 5 μm.

(B) Cells were treated with dsRNAs for indicated PP2A subunits and their mitotic cohesion were examined by ChX FISH ($n = 3, \geq 20$ cells per condition, mean ± SEM).

(C-E) Wdb-GFP-transfected cells were treated with control or Wapl dsRNA in the presence or absence of Dmt dsRNA and the cells were spun onto slide glasses after hypotonic treatment and

immunostained with anti-Dmt and anti-GFP antibodies. DNA was counterstained with DAPI. Magnified image of a chromosome is shown on the right. Bar: 5 μm (C). In each condition, centromeric/arm signal ratios of Wdb-GFP (green circle "cent." / dashed circle "arm" in C) were plotted against Dmt intensities (D) or only centromeric/arm signal ratios of Wdb-GFP were shown (E). Red or black bars denote the median, lower, and upper quartile values ($n \geq 32$ cells per condition; $^{****}P < 0.0001$, two-tailed Mann-Whitney *U*-test).

(F) $Dmt^{275\text{-}299}$ is required for association of Dmt with Wdb. Whole cell extracts from S2 cells expressing various N-terminus truncations and Wdb-mCherry were subjected to GFP-nanobody pull-down assay. Whole cell extracts (input) and pull-down fractions were immunoblotted with anti-GFP (Dmt-GFP) or anti-mCherry antibody. The amount of Wdb-mCherry bound to Dmt was significantly reduced in Dmt (ΔN299) compared with Dmt (ΔN274).

(G) Untransfected S2 cells (-) or Dmt (WT)- or Dmt (ΔPPB)-GFP-transfected cells were treated with control or Dmt dsRNA. Whole cell extracts from these cells were subjected to GFP-nanobody pull-down assay and the pull-down fractions were immunoblotted with Dmt and tubulin antibodies. Note that both WT and ΔPPB were overexpressed compared to with endogenous level.

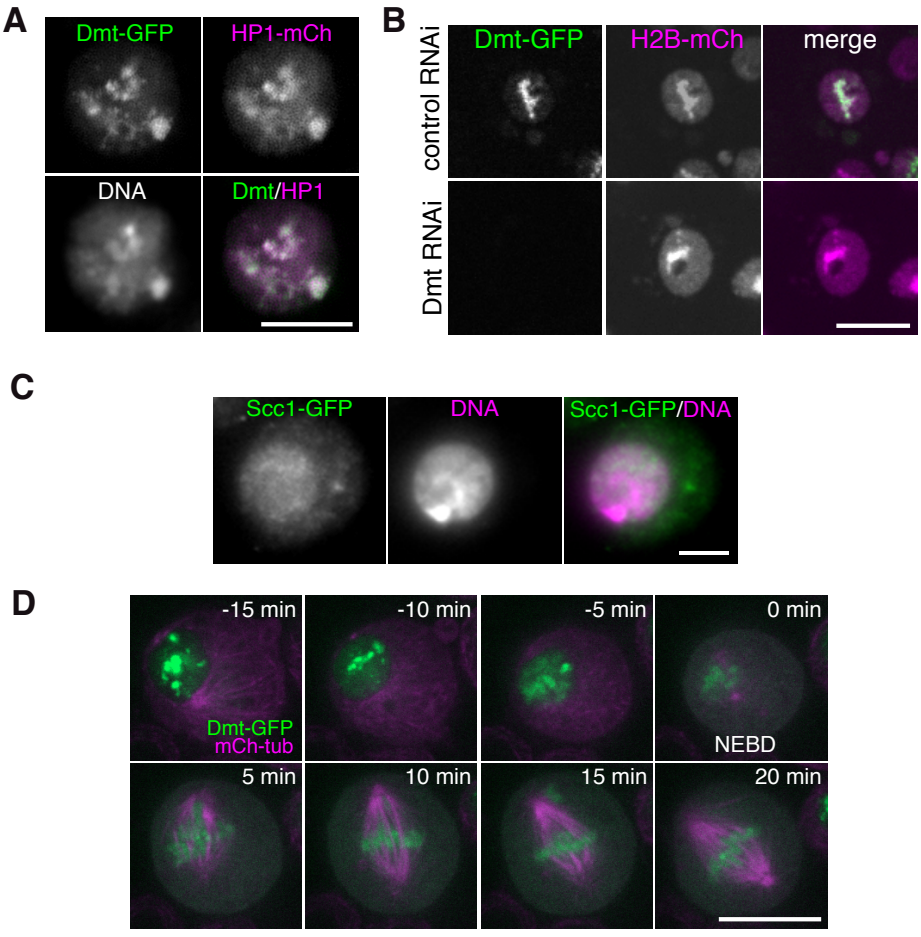
(H) Cells expressing Dmt (WT)- or Dmt (ΔPPB)-GFP were spun onto slide glasses after hypotonic treatment, and immunostained with anti-GFP antibody. DNA was counterstained with DAPI. Both Dmt (WT) and Dmt (ΔPPB) were localized to pericentromeres in mitosis. Bar: 5 μm.

Appendix Figure S6. Bub1 is not required for Dmt localization on heterochromatin in mitosis and interphase

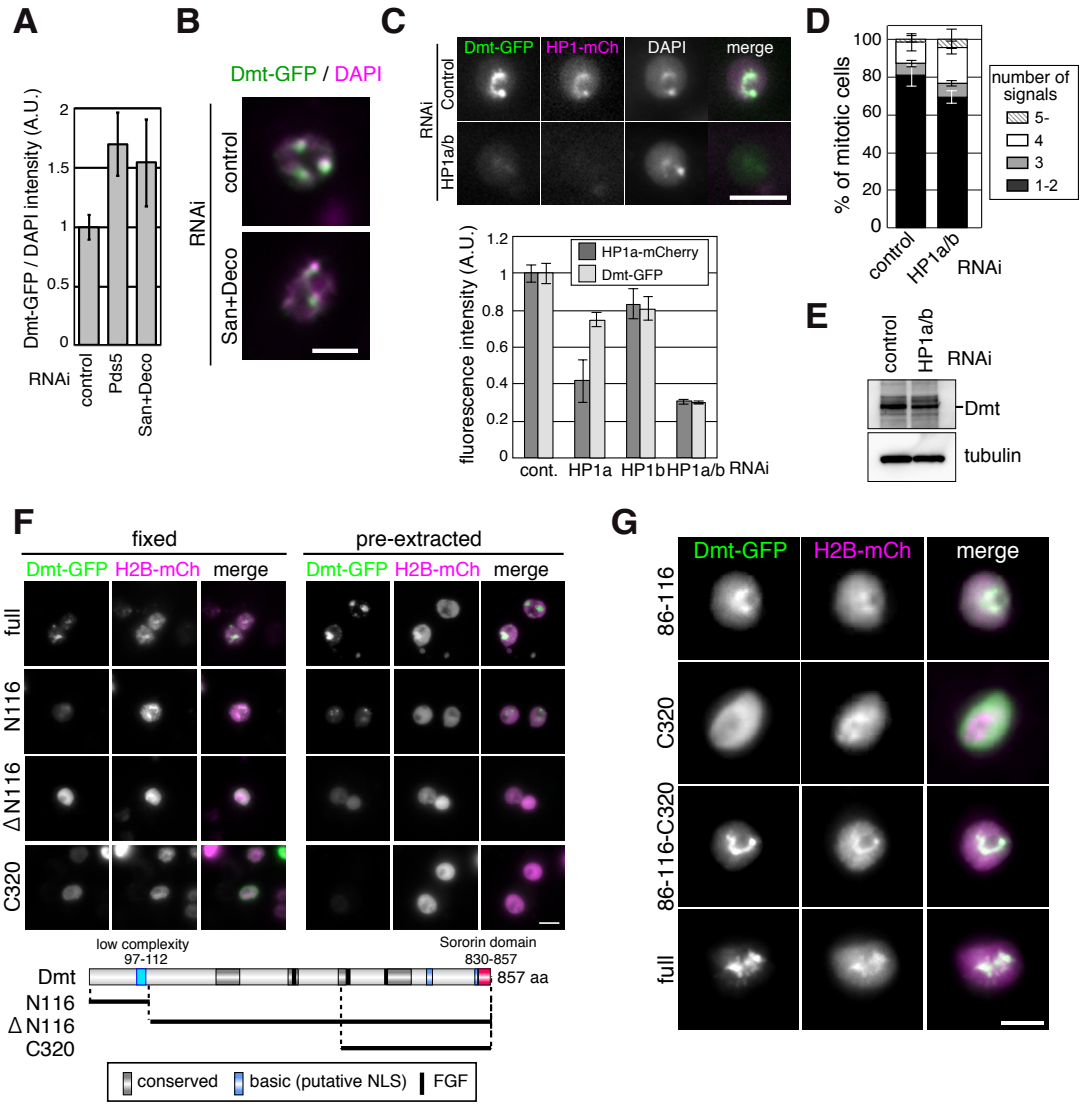
Control or Bub1 RNAi cells were spun onto slide glasses after hypotonic treatment and immunostained with anti-Dmt antibody. Mitotic chromosomes (A) and interphase nuclei (B) are shown. Note that cells with separated chromosomes were accumulated in Bub1 RNAi condition. DNA was counterstained with DAPI. Bar: 5 μm.

Appendix Figure S7. ChIP sequencing analysis of Dmt-GFP.

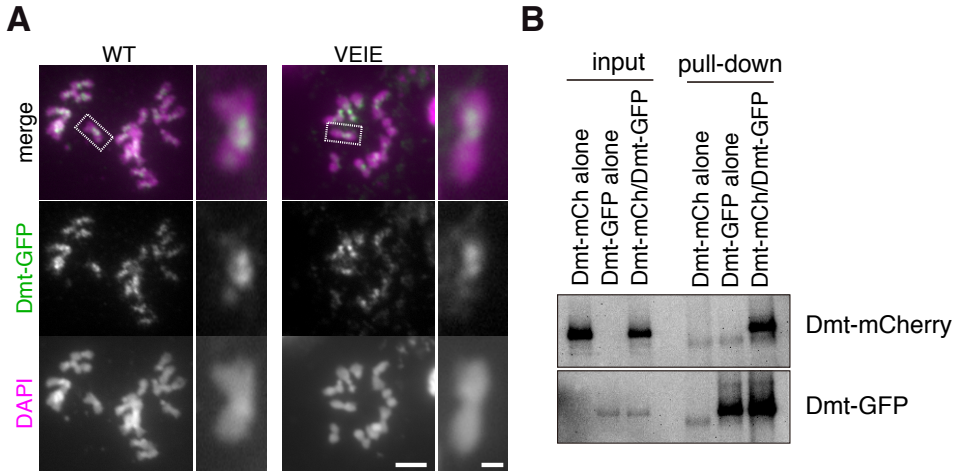
(A-C) Three representative loci are shown. Dmt-GFP was accumulated on loci where cohesin (Scm3) and/or H3K9me3 signals were enriched. ChIP-seq data of Smc3 (D.mel 3rd Instar Larvae nuclei) and H3K9me3 (D.mel Embryo 20-24h) were referred to modENCODE Genome-wide Chromatin Profiling (GSE20794). Note that several Dmt-GFP peaks are present in a region, where H3K9me3 is poorly accumulated (B).



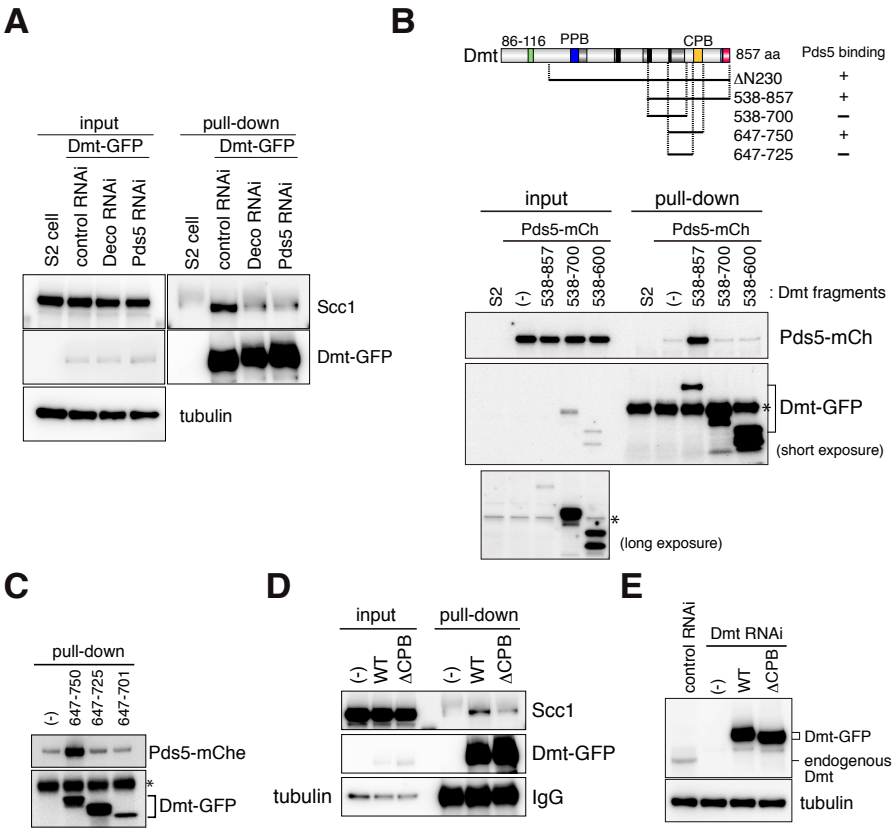
Appendix Figure S1



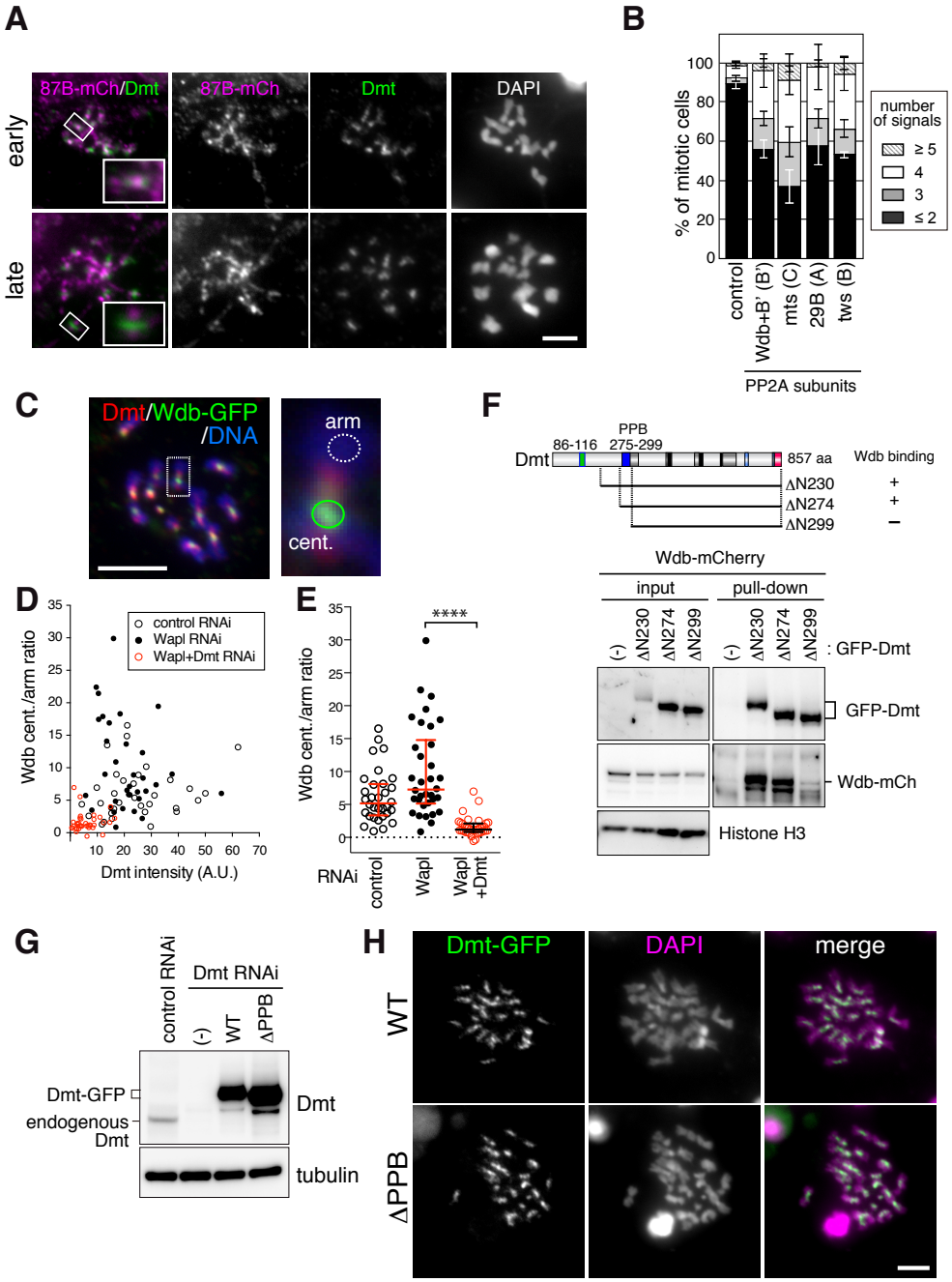
Appendix Figure S2



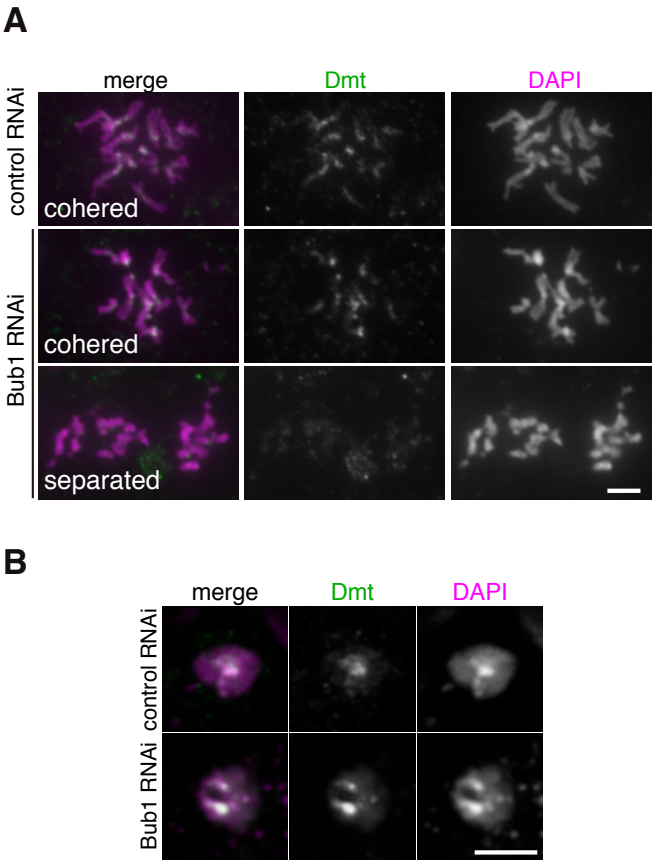
Appendix Figure S3



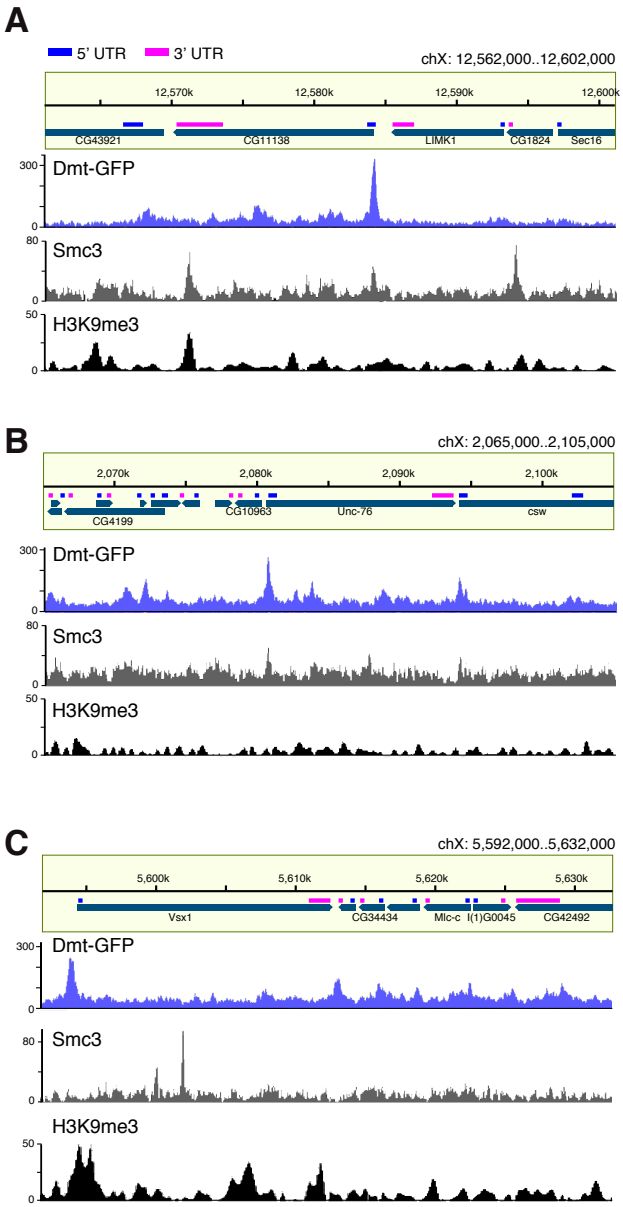
Appendix Figure S4



Appendix Figure S5



Appendix Figure S6



Appendix Figure S7

target gene	Sequence (5' to 3')
Pds5	AAGACACTAACTCATCTGGGGC ACCTACGCCCTTTTGTTCG
Rad21	ATACCAAAGAGAACGAGAAC CTGGGAATTTCATAGTTGTC
Deco	TACTACTTCCTGGGCTTTCTGG AAGGAACTCCTTCTTCCAGAGC
San	CTGACTAGGAGCAGCATCGAACTGG AAACAAATGTGAACTGTCGTGC
Cdh1	ATTCAGAACGGACTTGTTCTCC CACCGGATAATCAATACTTGGC
Control (pBluescript)	TAAATTGTAAGCGTTAATATTTTG AATTCGATATCAAGCTTATCGAT
Cdc27	AACAATAGCCTAAACAATGCCC TTGCATGGCGCCGATGTGCACGAG
Dmt-exon(M)	TCCTTATCGACCATTTTCGAGCC AACATGAAAGTATTGGGCAATGG
HP1a	AGACCCTGAGAGCTCGGCAAAGGTTTC AGACTTCATTCTCAGAGTACCAGGATAGGCGCTCTTC
HP1b	TAAAGCCGGTGTGTATGTATCC AATACGAATTCCGTCAATTTGG
Mei-S332	CTGCAAAACCATCGCCAGCG GGGCGTGACGGTGACTTGG
Wapl	ATGTCGCGCTGGGGCAAGAACA AAGGCTGTCAACTACC
Wdb	GAGGAACTGTGGAAGAAACTGC CCAGATTTCGGTTCTACCTCC
B'	AAAAAGCCGCTGATCTATCTCC AAGCCTACTTAAATTTTGGCCC

Appendix Table S1. Primers used for RNAi

For all primers, T7 RNA polymerase-binding sequence (TAATACGACTCACTATAGGG) was attached to 5' ends.

Protein name	Peptide
Protein dalmatian OS=Drosophila melanogaster GN=dmt PE=1 SV=1	192
ATP-dependent RNA helicase abstrakt OS=Drosophila melanogaster GN=abs	30
Histone H2A OS=Drosophila melanogaster GN=His2A:CG33856	21
FACT complex subunit spt16 OS=Drosophila melanogaster GN=dre4	18
Heterochromatin protein 1 OS=Drosophila melanogaster GN=Su(var)205	13
Pds5 OS=Drosophila melanogaster GN=pds5 PE=2 SV=1	12
Nuclear GTP binding protein OS=Drosophila melanogaster GN=Ns2	11
DNA-binding protein modulo OS=Drosophila melanogaster GN=mod	10
Origin recognition complex subunit 1 OS=Drosophila melanogaster GN=Orc1	9
D1 chromosomal protein, isoform C OS=Drosophila melanogaster GN=D1	9
Heterochromatin protein 5, isoform B OS=Drosophila melanogaster GN=HP5	8
Replication factor C 38kD subunit OS=Drosophila melanogaster GN=RfC38	7
Inner centromere protein OS=Drosophila melanogaster GN=Incenp	7
Serine/threonine-protein phosphatase alpha-2 isoform OS=Drosophila melanogaster GN=Pp1-87B	7
Isoform B of Borealin OS=Drosophila melanogaster GN=borr	7
Replication factor C subunit 3 OS=Drosophila melanogaster GN=RfC3	7
Serine/threonine-protein phosphatase PP2A 65 kDa regulatory subunit OS=Drosophila melanogaster GN=Pp2A-29B	7
Serine/threonine-protein phosphatase alpha-3 isoform OS=Drosophila melanogaster GN=Pp1-13C	6
Chromosome-associated protein, SMC3, OS=Drosophila melanogaster GN=Cap	6
Histone deacetylase complex subunit SAP18 OS=Drosophila melanogaster GN=Bin1	6
Argonaute 2, isoform E OS=Drosophila melanogaster GN=AGO2	5
Topoisomerase 1 OS=Drosophila melanogaster GN=Top1	4
Structural maintenance of chromosomes protein OS=Drosophila melanogaster GN=SMC1	4
FI11703p OS=Drosophila melanogaster GN=vtd	4
LD34181p OS=Drosophila melanogaster GN=SA	3
Aurora kinase B OS=Drosophila melanogaster GN=ial	2
Protein phosphatase PP2A 55 kDa regulatory subunit OS=Drosophila melanogaster GN=tws	2

Appendix Table S2. Mass Spectrometry analysis of Dmt-GFP binding proteins

Chromatin-bound Dmt-GFP was extracted and immunoprecipitated by anti-GFP antibody. The bound proteins were analyzed by mass spectrometry. Cohesin-related proteins, heterochromatin proteins, and serine/threonine-protein phosphatases were highlighted by red.